

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : G01N 21/64, 15/14	A1	(11) International Publication Number: WO 99/60383 (43) International Publication Date: 25 November 1999 (25.11.99)
(21) International Application Number: PCT/US99/10874 (22) International Filing Date: 17 May 1999 (17.05.99) (30) Priority Data: 60/085,545 15 May 1998 (15.05.98) US (71) Applicant (for all designated States except US): FLUORRX, INC. [US/US]; 979 Keystone Way, Carmel, IN 46032 (US). (72) Inventor; and (75) Inventor/Applicant (for US only): SZMACINSKI, Henryk [PL/US]; 979 Keystone Way, Carmel, IN 46032 (US). (74) Agent: KENEMORE, Max; FluorRx, Inc., 979 Keystone Way, Carmel, IN 46032 (US).		(81) Designated States: CA, JP, US, European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published With international search report.
(54) Title: IMPROVED PHASE ANGLE AND MODULATION OF FLUORESCENT ASSAY (57) Abstract <p>The presence or concentration of analytes in a sample can be assayed by observing with fluorescent lifetime techniques such as phase angle shift and modulation change the combined response to excitation of a fluorescent probe in the presence of the sample and a fluorescent material, the probe having a fluorescence intensity response to the presence or concentration of the analyte, the probe and the material having no substantial fluorescence lifetime change in response to the presence or concentration of the analyte and the material having a fluorescence lifetime in response to excitation that is different from the fluorescence lifetime of the probe.</p>		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece	ML	Mali	TR	Turkey
BG	Bulgaria	HU	Hungary	MN	Mongolia	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MR	Mauritania	UA	Ukraine
BR	Brazil	IL	Israel	MW	Malawi	UG	Uganda
BY	Belarus	IS	Iceland	MX	Mexico	US	United States of America
CA	Canada	IT	Italy	NE	Niger	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NL	Netherlands	VN	Viet Nam
CG	Congo	KE	Kenya	NO	Norway	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NZ	New Zealand	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	PL	Poland		
CM	Cameroon	KR	Republic of Korea	PT	Portugal		
CN	China	KZ	Kazakhstan	RO	Romania		
CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

Title

5

IMPROVED PHASE ANGLE AND MODULATION OF FLUORESCENT ASSAY

10

15

20

25

30

Description

5 At present, there is considerable interest and research activity in the field of chemical sensing. Rapid and continuous monitoring of many analytes (pH, pCO₂, O₂, metal ions, etc.) Is required in many areas of science, including analytical chemistry, biochemistry,
10 environmental sensing, clinical chemistry and industrial applications. Fluorescence-based sensing is one of the promising techniques because of fluorescence sensitivity, providing number of sensitive and specific fluorescent probes for a variety of analytes and their
15 fabrication with fiber optics.

 At present, most types of commercial fluorescence sensing devices are based on the standard intensity-based methods, in which the intensity of the fluorescence produced by the probe molecule changes in
20 response to the analyte of interest. These intensity changes can be induced by an analyte due to changes in extinction coefficient, changes in quantum yield, absorption and emission spectral shifts, or simply due to the inner filter effects. While intensity
25 measurements are simple and accurate in the laboratory, they are often inadequate in real-world situations. This is because the sample may be turbid, the optical surfaces may be imprecise and become dirty and optical alignment may vary from sample to sample. A significant
30 disadvantage of intensity based sensing is the problem of referencing the intensity measurements. The

fluorescence intensity measurement depends on the intensity of exciting light, the optical density at the excitation and emission wavelengths, the light loses in the optical path length, detector sensitivity and the concentration of the fluorophore. These difficulties with intensity-based sensing appear to be limiting the more widespread use of fluorescence for quantitative chemical sensing.

Recent advances in optoelectronic have now made possible a new type of fluorescence sensing. Instead of fluorescence intensities it is possible to measure fluorescence lifetime, particularly by the phase modulation method with a simple light sources. The advantages of lifetime-based sensing and the for a several mechanisms of analyte-induced changes in lifetime are reviewed elsewhere in detail (Szmajnski and Lakowicz, Topics in Fluorescence Spectroscopy, Vol 4, pp.295-334, Plenum Press, New York 1994). The preferred lifetime-based sensing technique is phase modulation, where analyte-induced changes in lifetime of the probe are measured by phase angle and modulation at single modulation frequency. The phase and modulation are related to the analyte concentration. A number of lifetime-sensitive probes have been characterized for several analytes such as pH, Ca^{2+} , Mg^{2+} , K^+ , Na^+ . Practically all of the known analyte lifetime-sensitive probes excluding the probes for O_2 sensing display short lifetimes, most often in the range of 1- 5 ns. Using probes with short lifetimes requires high modulation frequencies in the range of 50 - 300 MHz in order to obtain sufficient changes in phase and modulation for

analyte sensing. However, even though inexpensive light sources such as LED's can be modulated in that range of frequencies, the cost of phase modulation device is still sufficiently expensive to inhibit broad commercial use.

There is observed a significant effort in several laboratories to develop functional long lifetime probes having a fluorescence lifetime in the range above 100 ns. There is a large number of fluorophores that display long lifetime fluorescence, such as metal-ligand complexes based on ruthenium, rhenium, osmium, platinum or rhodium. Lifetimes as long as 100 μ s can be obtained using such probes. However, there are not known such metal-ligand complex-based probes that are sufficiently sensitive to analytes for practical use. Only O₂ probes based on metal-ligand complexes in which the mechanism of quenching is exploited are presently used widely. The advantage of longer lifetime probes is that sensing can be at a relatively lower modulation frequency, for example, in the range of 10-1000 kHz. A phase and/or modulation instrument based on the use of longer lifetime fluorescent materials, such as the metal-ligand complex based materials can be designed to use inexpensive components due mainly to the lower frequencies required.

It is among the objects of the present invention to overcome the disadvantages of the present technology.

It is one object to avoid the need for development and manufacture of relatively exotic long lifetime fluorescent materials such as those based on metal-ligand complexes.

It is another object of this invention to avoid the need for the use of high frequencies in phase-modulation assays, so that relatively less expensive lower frequency electronic components can be used.

5 It is yet another object of this invention is to measure phase and modulation in the low modulation frequencies using available (or designed) fluorophores in which the intensity is sensitive to the analyte of interest.

10 It is a further object of this invention to avoid the problems encountered in the past with intensity-based fluorescent assays due to ambient light, turbidity in the sample, the need for optical couplings, intensity dissipated in wave guides, and the like.

15 These and other objects are accomplished by the present invention, which makes use of the discovery that relatively low frequency phase and modulation techniques can be used to assay for analytes by employing, in combination, a fluorescent probe that shows a
20 fluorescent intensity response in the presence of the analyte but has a fluorescent lifetime that is substantially unaffected by the presence of the analyte and a fluorescent material having a fluorescent lifetime that is different from the fluorescent lifetime of the
25 probe, the fluorescent material being substantially unaffected in both fluorescence lifetime and fluorescence intensity by the presence of the analyte.

Although the mechanism by which this invention works is not fully understood at the present time, it is
30 believed that the phase angle and modulation of the sample depend on values of lifetime and fractional

intensities of components. The changes in phase angle and modulation can be as result of changes in fractional intensities without changes in the lifetime of both component. By mixing an analyte sensitive fluorophore (short lifetime or long lifetime) with the second fluorophore that is not analyte sensitive (long lifetime or short lifetime) and by correctly selecting the relative concentration of the fluorophores, the excitation wavelength, and the emission band observed, an analyte sensitive probe can be created. The expected analyte induced changes in phase angle and modulation can be as large as 90 degree and 1.0, respectively. To observe large changes in phase angle and modulation the modulation frequency can be at a lower range, determined by the long lifetime component.

The controlled mixing of two fluorophores allows using any intensity-based fluorophore regardless of its lifetime as a lifetime-based probe using a phase and modulation technique. The analyte-induced changes in fractional intensities of two components allow the determination of the analyte concentration from the phase and/or modulation at a single modulation frequency.

The properties, requirements, advantages and various applications of the present invention are discussed below with reference to the drawings.

Brief Description of the Drawings

Figs. 1a and Fig. 1b show the expected frequency responses of phase angle and modulation for long

lifetime and short lifetime fluorophores.

Fig. 2a and Fig 2b show the expected frequency responses of phase angle and modulation of fluorescence that consist of fraction of long lifetime and fraction
5 the short lifetime fluorescence.

Fig. 3a and Fig. 3b show the expected frequency responses of phase angle and modulation where the value of short lifetime is changed from 0.5 to 10 ns in several steps.

10 Fig. 4a and Fig 4b show the expected frequency responses of phase angle and modulation where the value of long lifetime is 100, 500, and 5000 ns and short lifetime fluorescence of 10 ns.

Figs. 5a, 5b, 6 and 7 illustrate Example 1.

15 Figs. 8, 9 and 10 illustrate Example 2.

Figs. 11, 12, 13, 14 and 15 illustrate Example 3.

Detailed Description

Referring again to Figs. 1a and 1b, two distinct
20 ranges of modulation frequencies are needed to measure the short lifetime and to measure long lifetime fluorescence. In order to measure the lifetimes shorter than 5 ns that display most of organic fluorophores, high modulation frequencies are required in order of 100
25 MHz. This is normally achieved by an expensive phase-modulation fluorometers which are commonly used in research labs. Long lifetime fluorescence requires low modulation frequencies in the range of 100 kHz. The design of a phase-modulation instrument for low
30 modulation frequencies is less expensive and can provide higher accuracy of measurements that similar devices

with high frequency. However, there are not presently widely available lifetime-sensitive fluorescence sensors that display long lifetimes other than those employing metal-ligand complexes and used in oxygen sensing as described above.

Figs. 2a and Fig 2b show the expected frequency responses of phase angle and modulation of fluorescence that consist of a fraction of long lifetime and a fraction the short lifetime fluorescence. The values from 0 to 1 represent the fractional intensity of short lifetime fluorescence in the measured signal. There are observed great changes from 0 to about 90 degrees in phase angle and from 1 to 0 in modulation values upon changes of fractional contribution of fluorescence from both fluorophores. In addition the steeples part of the modulation value is equal the fractional intensity of short lifetime fluorescence. Thus fractional-dependent phase angle and/or modulation can be used to measure the intensity of a desired fluorophore in the sample using in most cases only one modulation frequency. The changes in fractional intensity can be induced by the analyte; (1) by affecting the absorption spectra (extinction coefficient and/or spectra shift), (2) by affecting the emission spectra (quantum yields and/or spectra shifts). There are many possibilities to optimize such a sensor probe by the choice of excitation wavelength, emission band and relative concentration of used fluorophores.

Referring again to Figs. 3a and 3b, the fractional intensity of the short fluorescence lifetime component in each case is the same for 0.15 in Fig. 3a and 0.5 in Fig.3b. The important observations from these figures

are that the phase angle and modulation below certain frequency are not sensitive to the value of short lifetime fluorescence in the sample. This is similar to the gating technique in the pulse method where, by applying a certain delay after pulse excitation, only the signal from long lifetime fluorescence is detected. In the phase-modulation technique it is impossible to measure only long the lifetime component. Analytical methods have been developed for background correction in phase-modulation fluorometry based on the measurements of the background sample or based on known intensity decay of background and its contribution in the sample signal. In the case presented in Fig. 3a and 3b the desired intensity of the long lifetime component can be obtained by measuring the phase and/or modulation at single modulation frequency regardless of intensity decay of the background or autofluorescence or scattered light until the mean lifetime is short enough compared to a long lifetime fluorophore. This feature can be used also to determine the anisotropy of the long lifetime component in turbid media with scattered light or with background fluorescence having a known value of anisotropy. This may find immediate application in detecting binding of high molecular weight macromolecules labeled with a metal-ligand complexes or in immunoassays. It is also important for chemical sensing that changes in the lifetime due to presence of an analyte for short lifetime indicators have no effect of fractional intensity and thus on sensing of analyte concentration.

Referring again to Figs 4a and 4b, the most

important observation is from Fig 4b where the value of steeples part of modulation reflect the fractional intensity of the short lifetime component regardless on the value of the long lifetime component. Also, it is
5 important that the choice of low modulation frequency depends mostly on the value of the long lifetime component but not on the value of short lifetime component.

Examples

10

The present invention is further illustrated with reference to the following examples. Example 1 demonstrates the phase and modulation sensitivity when the fractional intensity of sample is varied by various
15 relative concentrations of two dyes in the sample.

Example 2 demonstrates the possibility to determine the intensity of flurophore of interest in presence of various amoun of background or autofluorescence from the solvent.

20 Example 3 demonstrate how the sensing probe can be created when pH induced changes in fractional intensities of a probe contained pH intensity sensitive indicator and long lifetime fluorophore can be measured by phase angle and modulation.

25

Example 1

Two fluorophores have been chosen, one with a long lifetime fluorescence from metal-ligand complexes like $[\text{Ru}(\text{bpy})_2\text{dcbpy}]^2$ with a lifetime in glycerol of 1060 ns
30 and the second with short lifetime like many organic fluorophores Texas Red Hydrazide with a lifetime of 3.4

ns in glycerol. The two dyes were mixed at various relative concentrations to induce the various fractional intensities in the sample

Fig. 5a show the absorption spectra of long
5 lifetime fluorophore $[\text{Ru}(\text{bpy})_2\text{dcbpy}]^{2+}$ and short lifetime fluorophore Texas Red Hydrazide (TRH) (solid lines and their mixture at concentrations specified in Figure. It is shown that any excitation wavelength shorter than about 640 nm will excite both fluorophores. The
10 resulting fractional intensities from both fluorophores will be strongly dependent on the choice of excitation wavelength. One can imagine that value of extinction coefficient or shift in absorption spectrum will result in changes of fractional intensities that can be
15 monitored with phase and/or modulation measurements. One excitation wavelength has been chosen as 488 nm (Argon-ion laser). The total concentration of dyes were low to avoid the inner filter effects. The changes in absorption was induced by using various concentration
20 combination of both fluorophores.

Fig. 5b shows the emission spectra of
[$\text{Ru}(\text{bpy})_2\text{dcbpy}]^{2+}$ and TRH at one selected concentration combination. The emission spectra overlap well and for phase and modulation measurements we used the long pass
25 filter above 550 nm.

Fig. 6 show the frequency responses of phase angle for long lifetime fluorophore $[\text{Ru}(\text{bpy})_2\text{dcbpy}]^{2+}$ with a lifetime of 1060 ns and the short lifetime TRH of 3.4 ns when mixed together at a specified relative
30 concentrations from 0 to 12.8. The obtained values for fractional intensities are in good agreement with those

expected from steady-state measurements of full emission spectra. These experimental data are related to those simulated and discussed in Fig. 2a.

Fig. 7 show the frequency responses of modulation for long lifetime fluorophore $[\text{Ru}(\text{bpy})_2\text{dcbpy}]^{2+}$ with a lifetime of 1060 ns and the short lifetime TRH of 3.4 ns when mixed together at a specified relative concentrations from 0 to 12.8. These experimental data confirm that presented and discussed in Fig. 2b.

10

Example 2

The purpose of this example was to demonstrate the calculation of intensity of long lifetime fluorophore in presence of background fluorescence from the solvent. Long lifetime fluorophore was the same as in Example 1. $[\text{Ru}(\text{bpy})_2\text{dcbpy}]^2$ with a lifetime in glycerol of 1060 ns. The glycerol (from Fluka) displayed a background fluorescence that overlaps with the emission of ruthenium. In many applications the requirements are for very low dye concentration which poses the difficulties for increased background corrections. The increased contribution of background fluorescence from solvent was obtained by the dilutions of the ruthenium sample with glycerol.

Fig. 8 show the emission spectra of ruthenium with decreased concentrations and also background fluorescence from used glycerol. The fractional intensity of glycerol calculated by integrating the spectra are following: 0.108, 0.379, 0.757, and 0.886 at ruthenium concentration of 740, 150, 29 and 6 nM.

30

Fig. 9 show frequency responses of phase angle of

the samples with increased contributions of background fluorescence. The obtained values are in good agreement with those from steady-state measurements. The small difference are because of different excitation sources (xenon lamp and monochromator in steady-state, and Ar-ion laser in phase-modulation measurements). It should be noted that phase angle is related only to fractional intensity at modulation frequencies lower than 1 MHz. The glycerol displayed a complex intensity decay with a mean lifetime shorter than 3.5 ns. These experimental data confirm that presented in Fig 3a where the short lifetime component do not contribute to changes in phase angle for certain low modulation frequencies.

Fig 10 show frequency responses of modulation of the samples with increased contributions of background fluorescence. The steeples part of modulation indicate good separation between the fluorescence of ruthenium and that of glycerol and can be easy used to determine the absolute intensity of the ruthenium. These results confirm that discussed in Fig. 3b.

Example 3

The goal of this example is to demonstrate the great opportunity of designing the fluorescence probe for measuring a large variety of chemical species where the change in fluorescence intensity can be obtained. For example we have chosen a pH intensity sensitive indicator Naphtofluorescein and the second dye with a long lifetime $[\text{Ru}(\text{phn})_3]^{2+}$. The Naphtofluorescein as most of fluorescein dyes display pH sensitive absorption spectrum and decreased fluorescence quantum yield at

lower values of pH. To demonstrate the practical use of such sensor we used inexpensive blue LED as a excitation source.

Fig. 11 shows the emission spectra of a mixture of ruthenium and Naphtofluorescein at various values of pH. The increased pH values affect the fractional intensities from both of dyes which is displayed as decreased fluorescence from the ruthenium and increased contribution from the Naphtofluorescein. The fractional intensities in the sample can be selected by the cutt off filter or by band pass filter. We have chosen use long pass filter above the 595 nm. The excitation source was a blue LED with a maximum intensity at 475 nm.

Fig. 12 shows the frequency responses of phase angle of such pH sensor. There are observed remarkably large changes in phase angle at modulation induced by the pH of a sample. The pH phase-based sensing can be performed at low modulation frequencies in spite of very short lifetime of Naphtofluorescein of about 0.45 ns frequencies below 10 MHz.

Fig. 13 shows large changes in modulation induced by pH of the sample. There is a wide range of modulation frequencies where modulation value is related only to the pH value even not to modulation frequency. This is because the difference in lifetimes of ruthenium and Naphtofluorescein is very large about 1000-fold. It is again important to note that long lifetime value determines the useful low modulation frequency for sensing.

Fig. 14 shows pH-dependent phase angle for several modulation frequencies. It should be noted the magnitude

of phase angle changes up to 69 deg (see values in the brackets) . This is remarkably pH sensor, which allows measurements the pH changes as small as of 0.0035 of pH unit assuming that phase angle can be measured with an
5 accuracy of 0.1 deg (from curve at 2200 kHz in the range from pH 6 to 8) . Also choosing the modulation frequency allow to shift the apparent pKa, in presented case from 6.41 to 7.24.

Fig. 15 shows pH-dependent modulations for several
10 modulation frequencies. The pH induced changes in modulation (values in the brackets) are large and significantly depends on the choice of modulation frequency. The apparent pKa is slightly dependent on modulation frequency.

15 The above description is intended to be illustrative of the present invention, which is intended to be limited only by the claims that follow.

20

25

30

5

Claims

10 What is claimed is:

1. A method for performing an assay for the presence
or concentration of an analyte in a sample, the
15 method comprising:
 - a. contacting the sample with a fluorescent probe
that, when in the presence of the analyte and
illuminated with activating radiation, emits
20 fluorescent radiation at an intensity that is
related to the presence or concentration of
the analyte, the fluorescent radiation having
a first lifetime that is substantially
unchanged by the presence or concentration of
25 the analyte in the sample;
 - b. illuminating with modulated activating
radiation of one or more frequencies or
30 amplitudes, or both, the fluorescent probe and
a fluorescent material that emits, in response

to said activating radiation, fluorescent radiation having a second fluorescent lifetime that is substantially unchanged by the presence or concentration of the analyte and that has an intensity that is substantially unchanged by the presence or concentration of the analyte and wherein the second fluorescent lifetime is longer or shorter than the first fluorescent lifetime;

c. sensing change in phase angle or modulation, or both, of the mixed emissions from the fluorescent probe and fluorescent material upon such contacting; and

d. determining from such change the presence or concentration of the analyte.

2. The method of Claim 1 wherein the sample is contacted by the fluorescent material.

3. The method of Claim 1 wherein the sample is contacted with a matrix material that supports the fluorescent probe and, optionally, the fluorescent material.

4. The method of Claim 1 wherein the sample is mixed

with the fluorescent probe and, optionally, with the fluorescent material.

5. An assay device for use in determining the presence or concentration of an analyte in a sample, the device comprising
 - a. a fluorescent probe that, when in the presence of the analyte and illuminated with activating radiation, emits fluorescent radiation at an intensity that is related to the presence or concentration of the analyte, the fluorescent radiation having a first lifetime that is substantially unchanged by the presence or concentration of the analyte in the sample;
 - b. a fluorescent material that emits, in response to said activating radiation, fluorescent radiation having a second fluorescent lifetime that is substantially unchanged by the presence or concentration of the analyte and that has an intensity that is substantially unchanged by the presence or concentration of the analyte and wherein the second fluorescent lifetime is longer or shorter than the first fluorescent lifetime; and
 - c. a support member adapted for supporting the probe and, optionally, the material in contact with the sample during illumination of the probe and the

material by the activating radiation.

6. The device of Claim 5 wherein the support member
5 comprises a polymeric matrix.

7. An assay composition for use in assaying for the
presence or concentration of an analyte in a
10 sample, the composition comprising:

a. a fluorescent probe that, when in the presence
of the analyte and illuminated with activating
15 radiation, emits fluorescent radiation at an
intensity that is related to the presence or
concentration of the analyte, the fluorescent
radiation having a first lifetime that is
substantially unchanged by the presence or
20 concentration of the analyte in the sample; and

b. a fluorescent material that emits, in response
to said activating radiation, fluorescent
25 radiation having a second fluorescent lifetime
that is substantially unchanged by the presence
or concentration of the analyte and that has an
intensity that is substantially unchanged by the
presence or concentration of the analyte and
30 wherein the second fluorescent lifetime is
longer or shorter than the first fluorescent

lifetime.

8. A kit for use in performing an assay for the presence or concentration of an analyte, the kit comprising:

- a. a container containing a fluorescent probe that, when in the presence of the analyte and illuminated with activating radiation, emits fluorescent radiation at an intensity that is related to the presence or concentration of the analyte, the fluorescent radiation having a first lifetime that is substantially unchanged by the presence or concentration of the analyte in the sample; and
- b. a container containing a fluorescent material that emits, in response to said activating radiation, fluorescent radiation having a second fluorescent lifetime that is substantially unchanged by the presence or concentration of the analyte and that has an intensity that is substantially unchanged by the presence or concentration of the analyte and wherein the second fluorescent lifetime is longer or shorter than the first fluorescent lifetime.

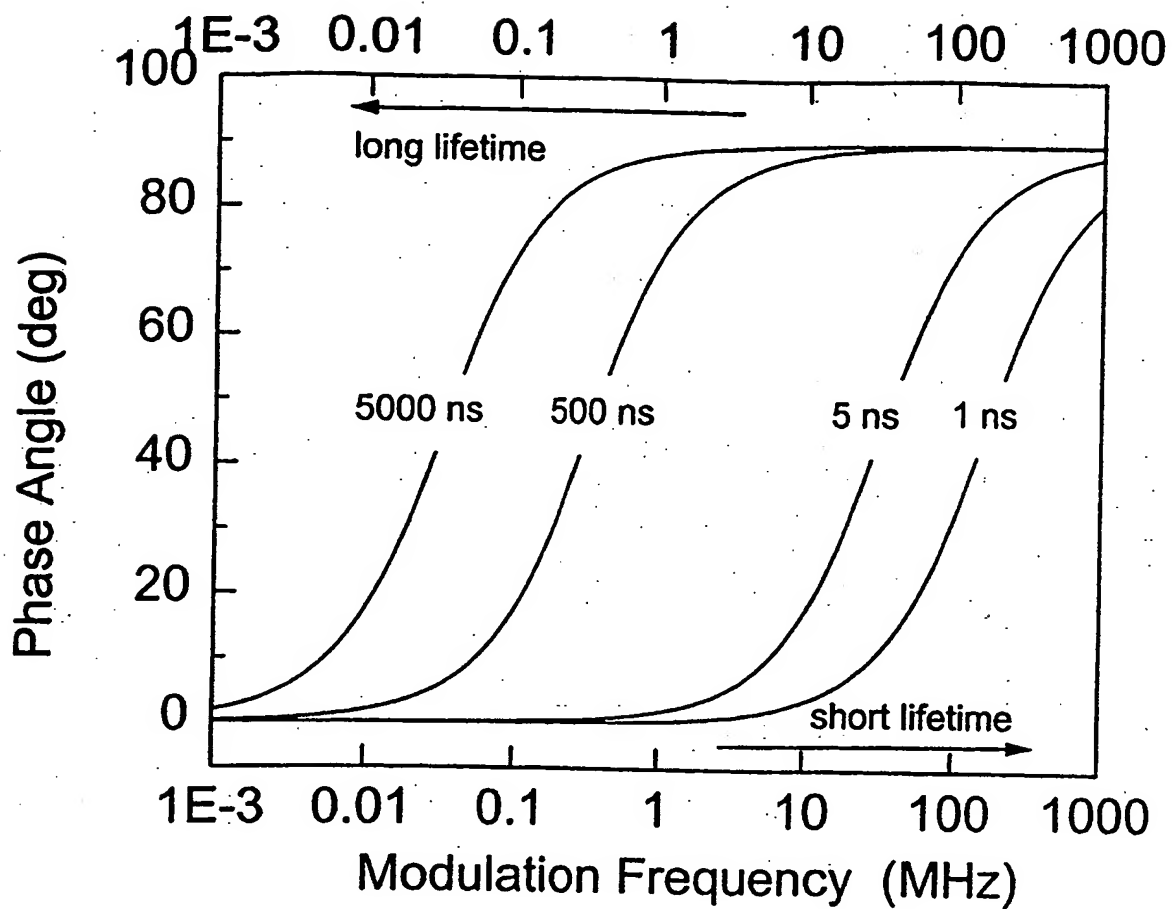


Fig. 1a

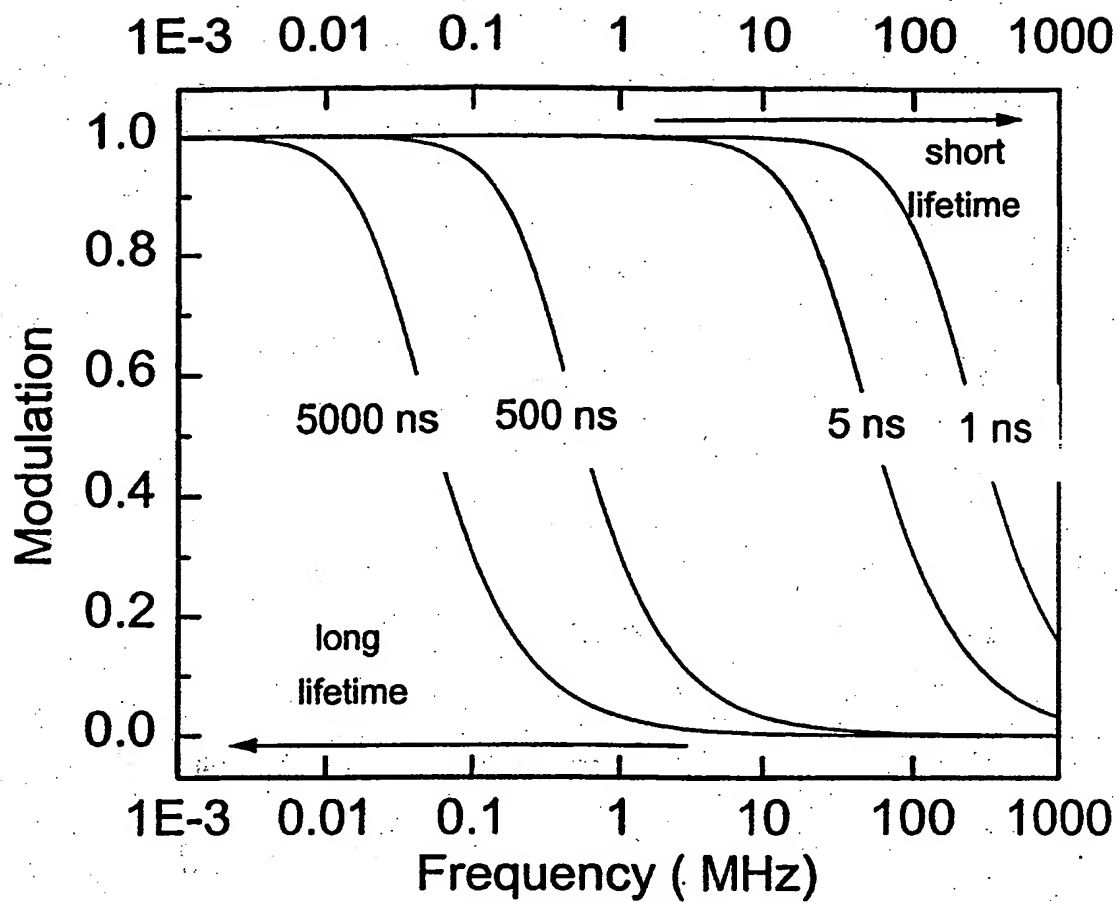


Fig. 1b

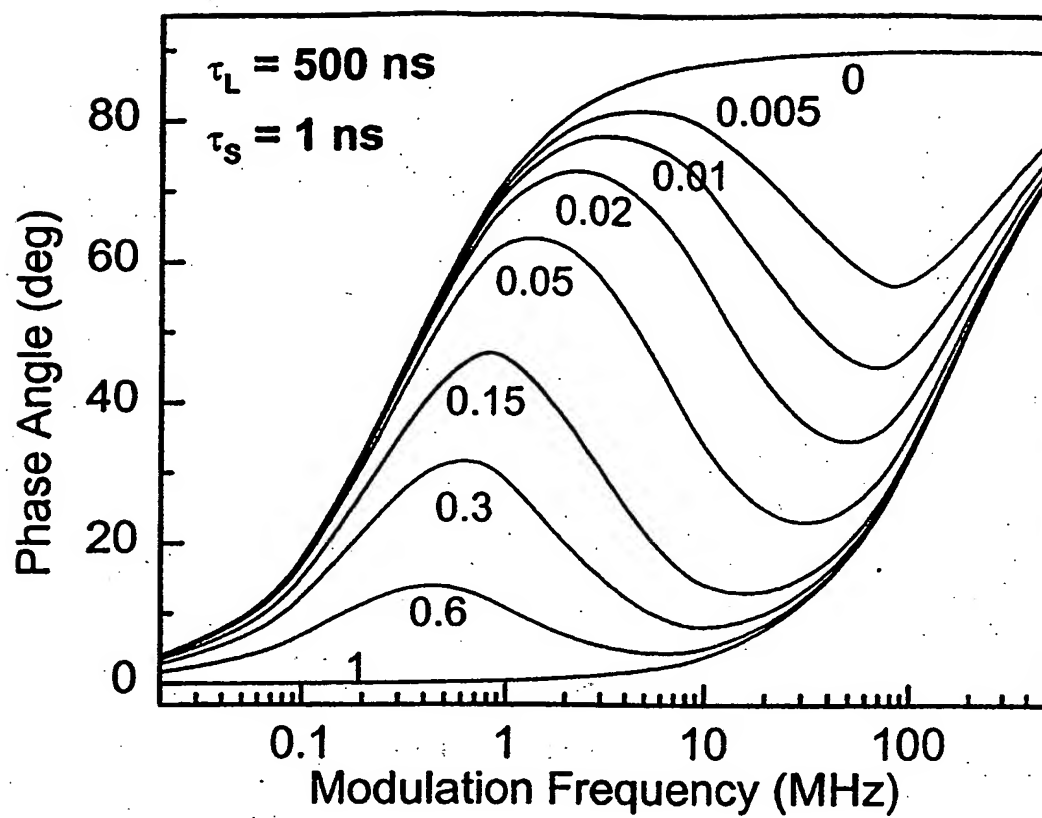


Fig. 2a

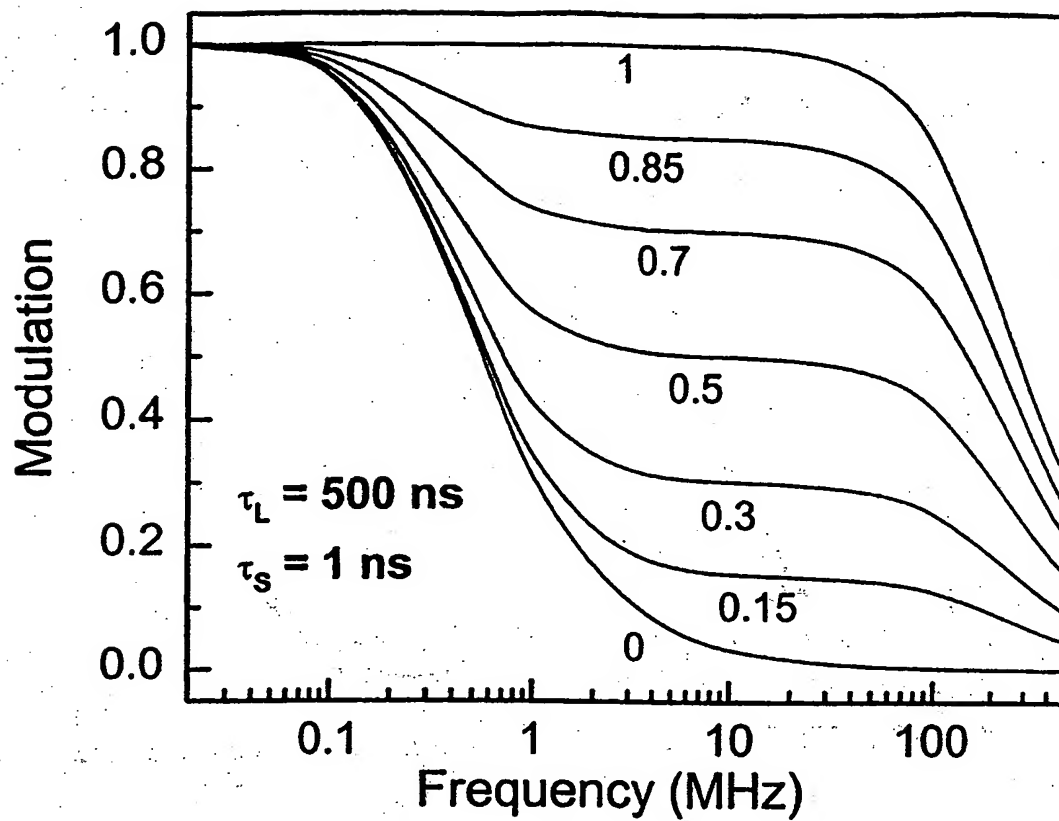


Fig. 2b

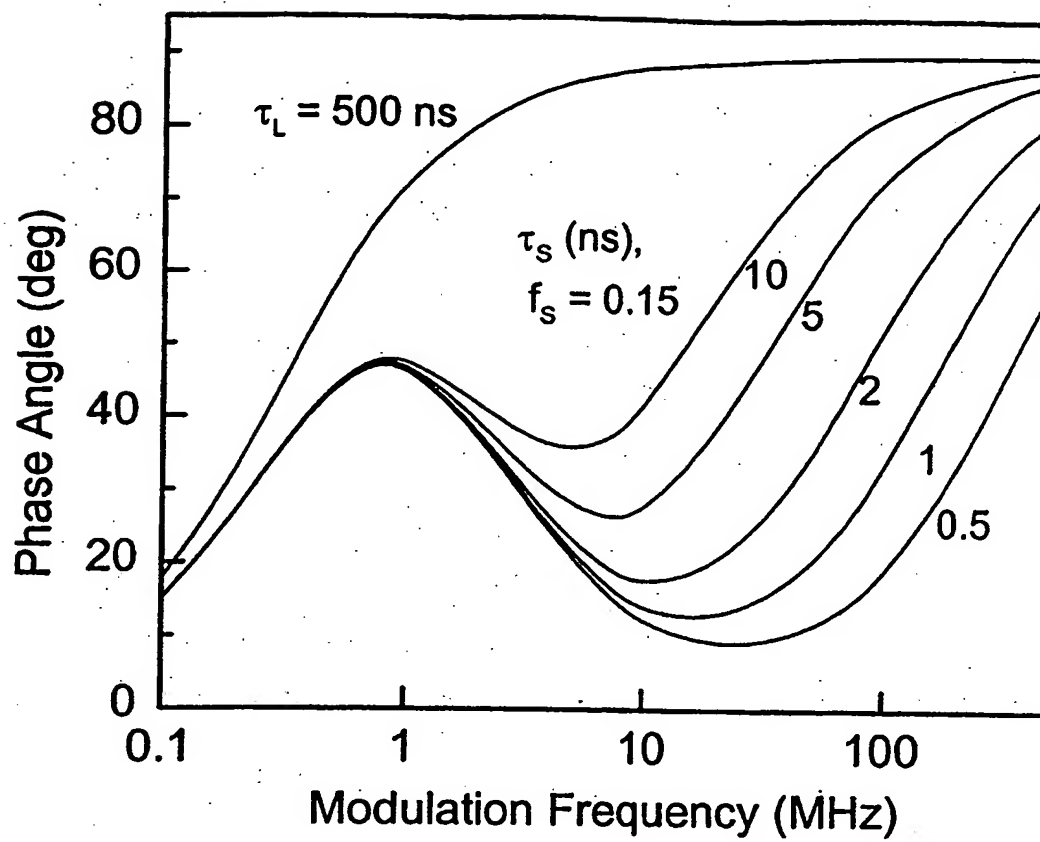


Fig. 3a

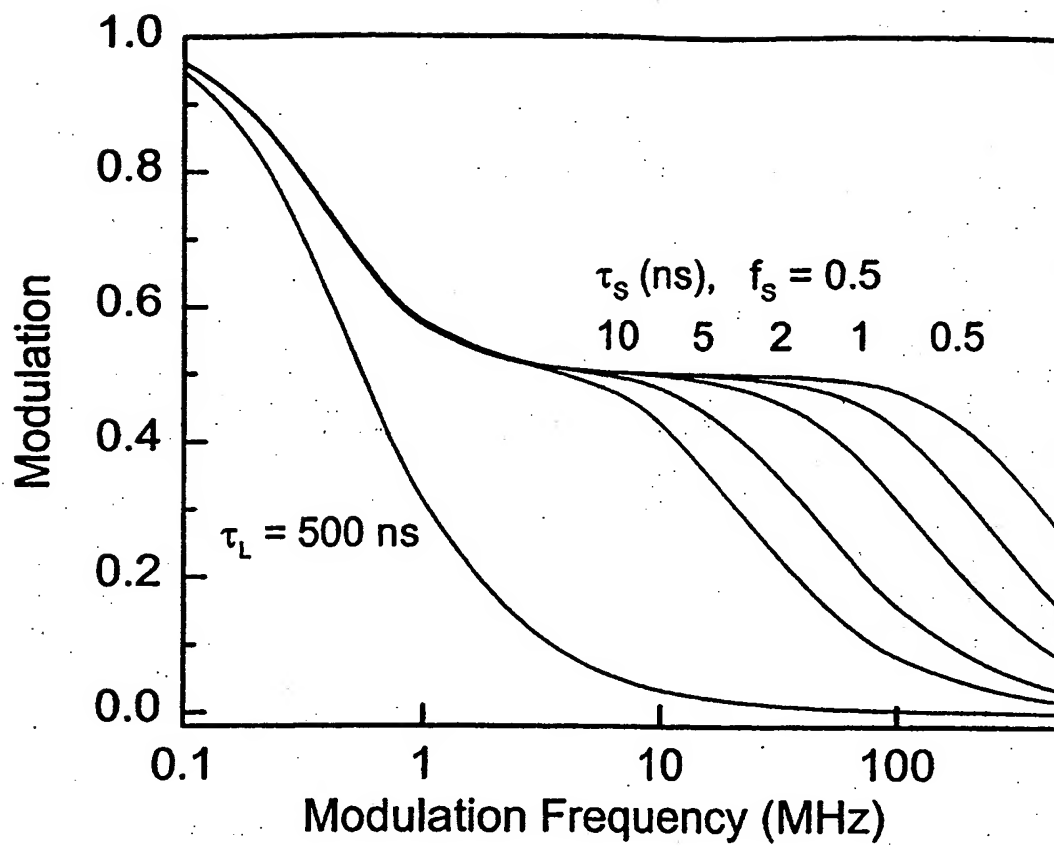


Fig. 3b

7/20

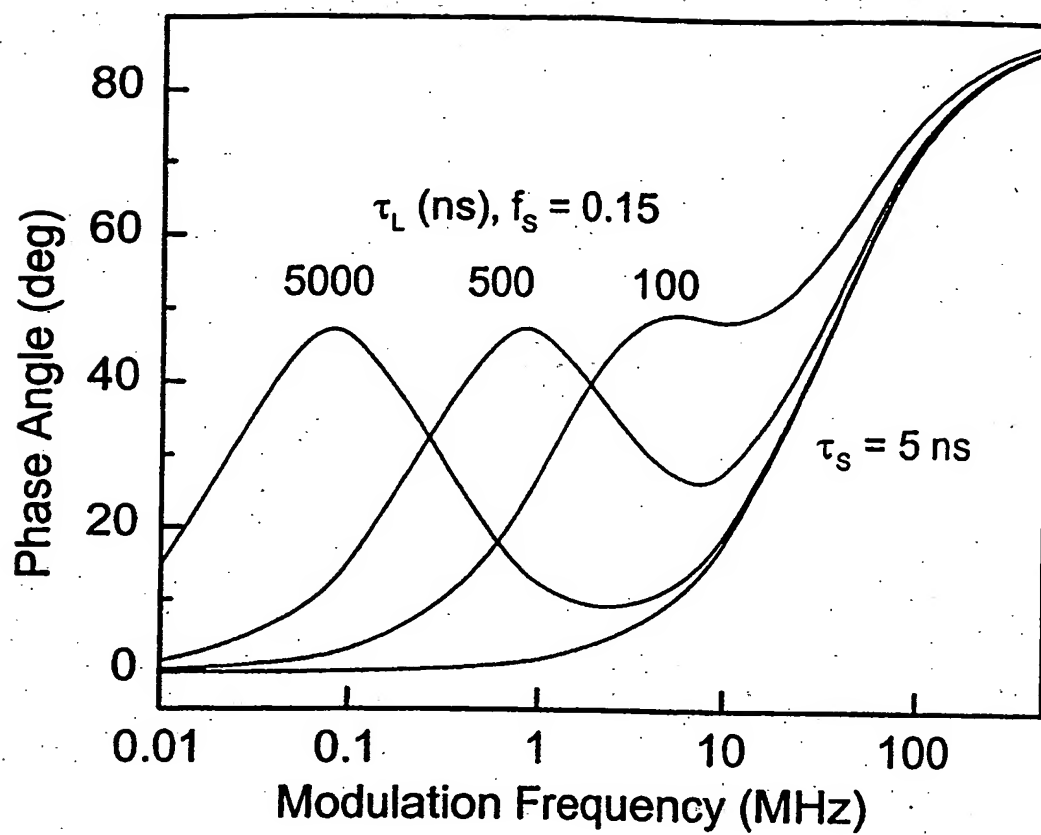


Fig. 4a

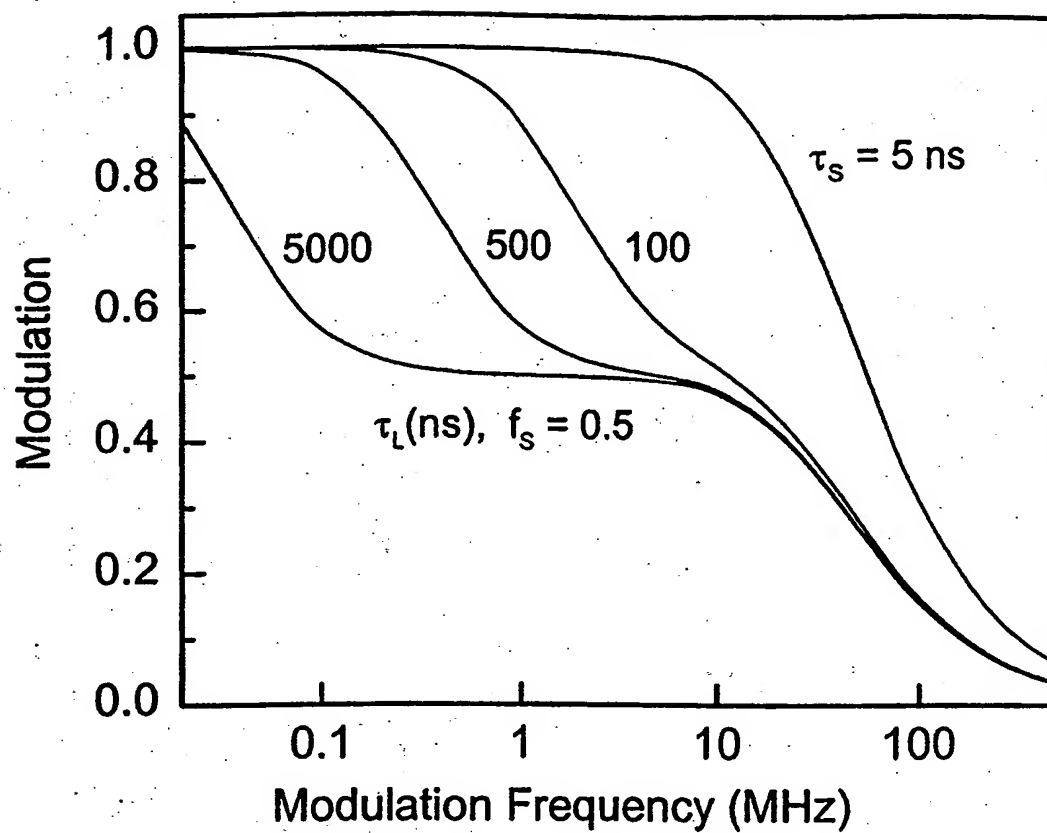
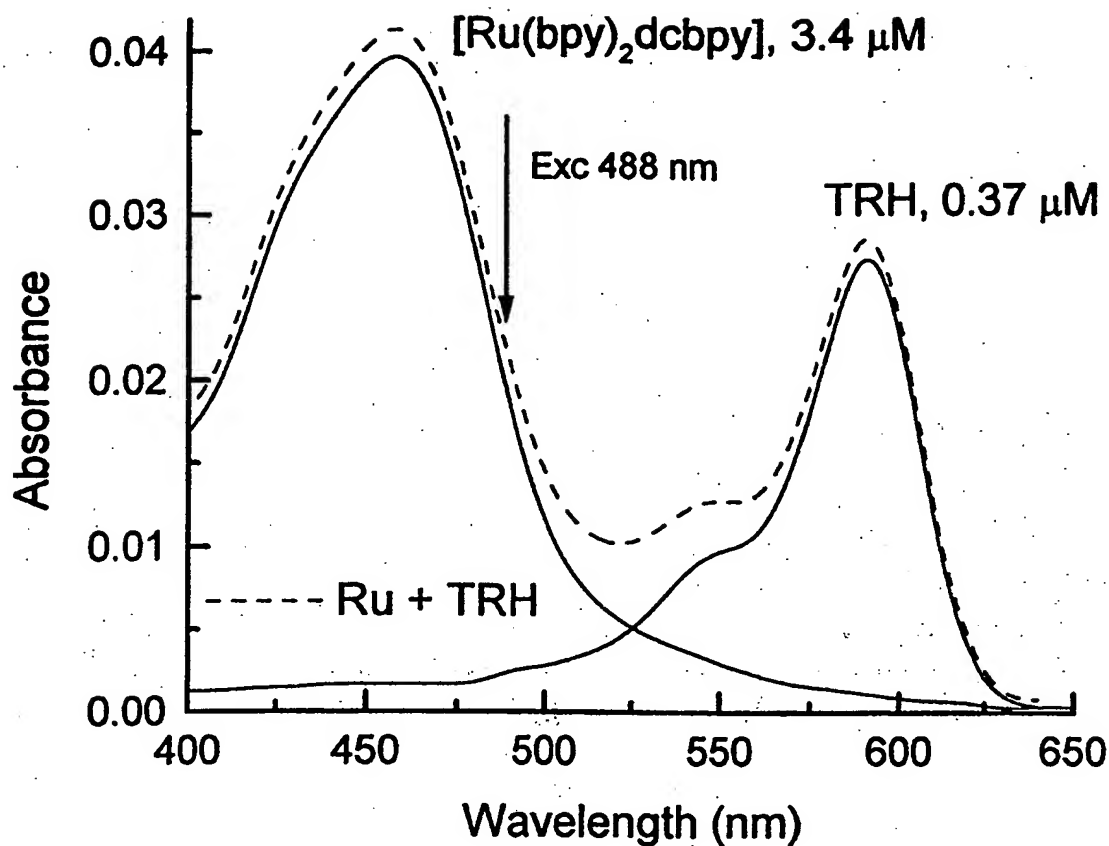


Fig. 4b

9/20



Serie B $OD_{488} = 0.21$ $TRH = 0.0024$ (488 nm)

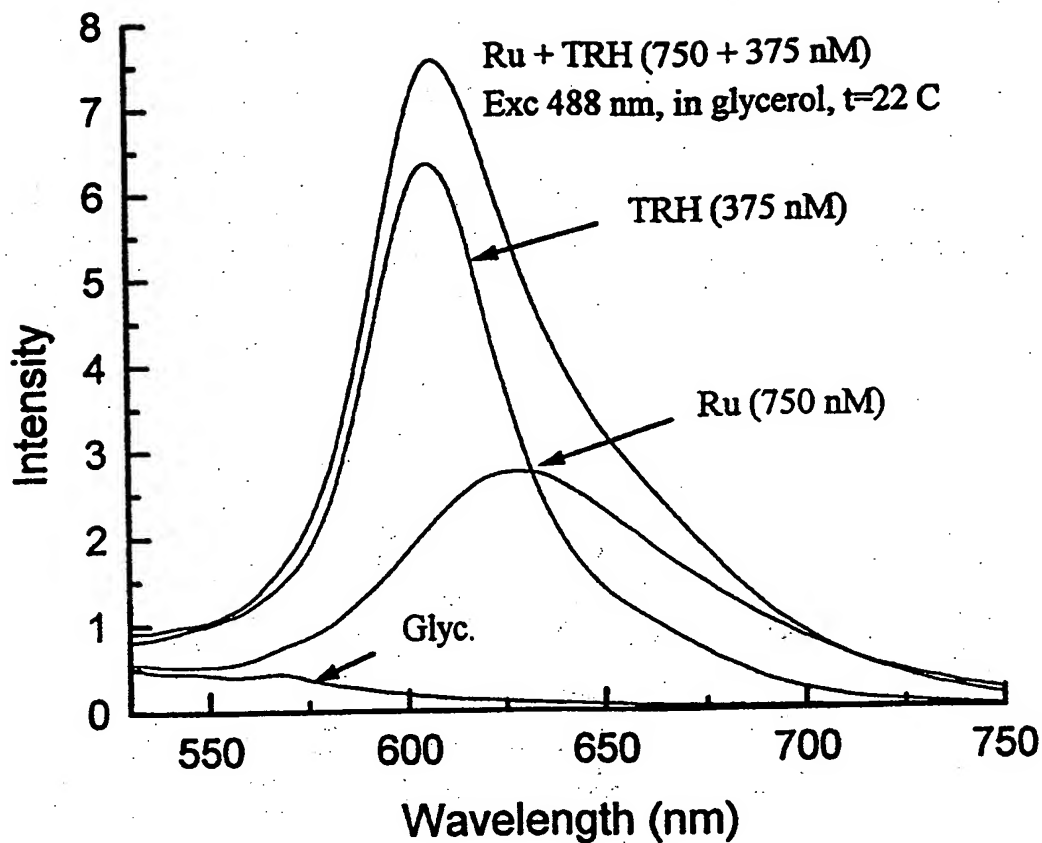
Dyes were in glycerol (Fluka).

Concentration calculated base on extinction coefficients :

Ru - 12,000 $M^{-1}cm^{-1}$, [Evald]

TRH- 80,000 $M^{-1}cm^{-1}$ [MP]

52.



Fractional intensity in the mixture (serie C):

glyc. - 0.065

Ru - 0.439

TRH - 0.613

$$\left. \begin{array}{l} .417 \\ .587 \end{array} \right\} \begin{array}{l} OD = 0.0042 \\ = 0.0024 \end{array} \left| \begin{array}{l} \text{Int } 1.41 \times \\ OD 0.57 \times \end{array} \right\} \phi = 2.4$$

If separately it t is about 11% more than in mixture.

S &

11/20

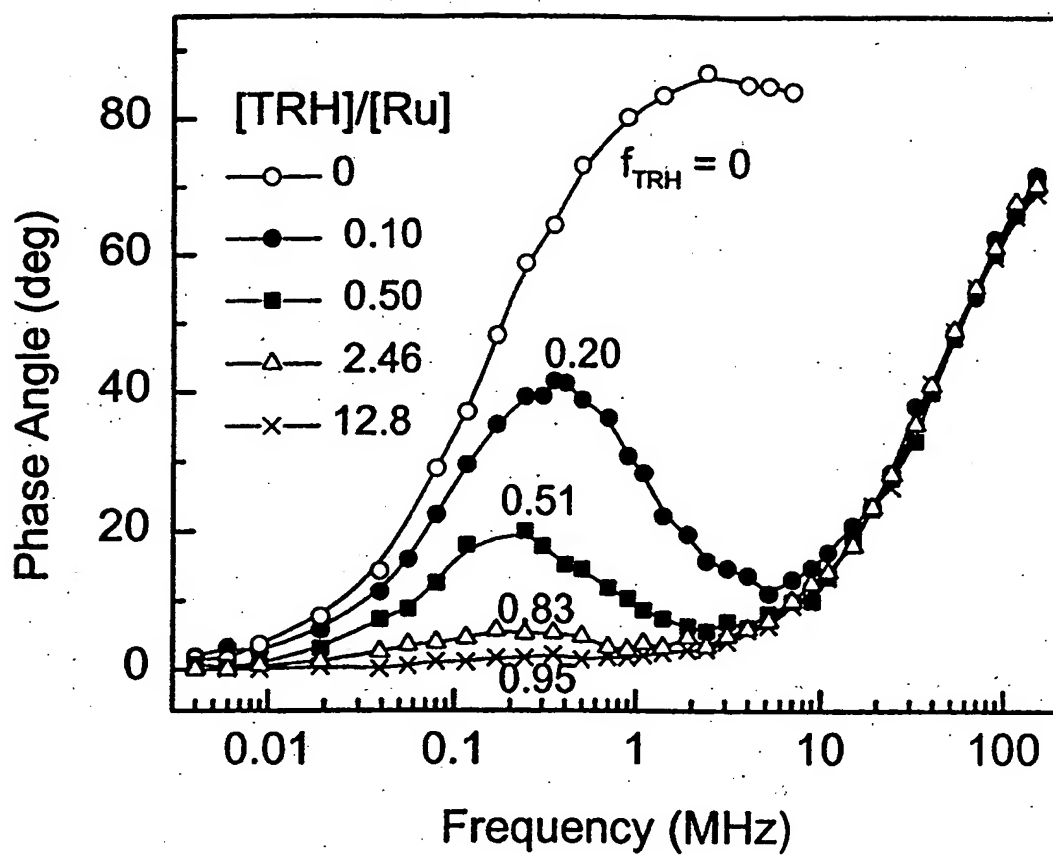


Fig. 6

12/20

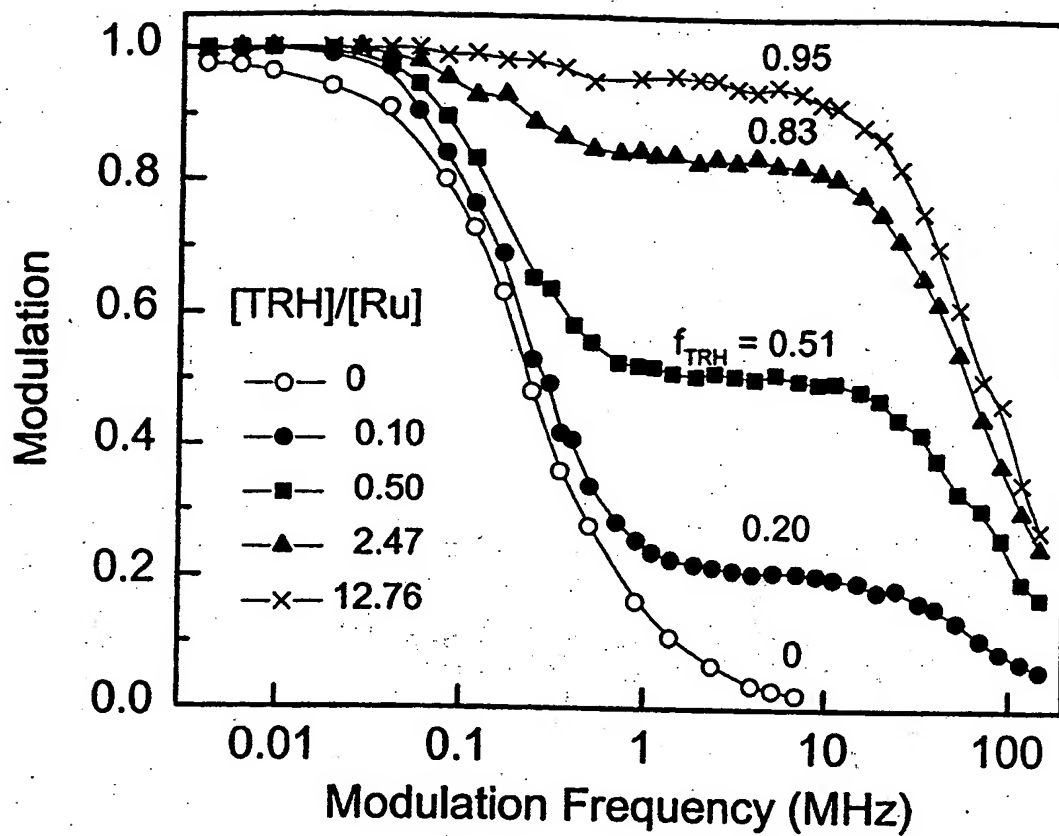


Fig. 7

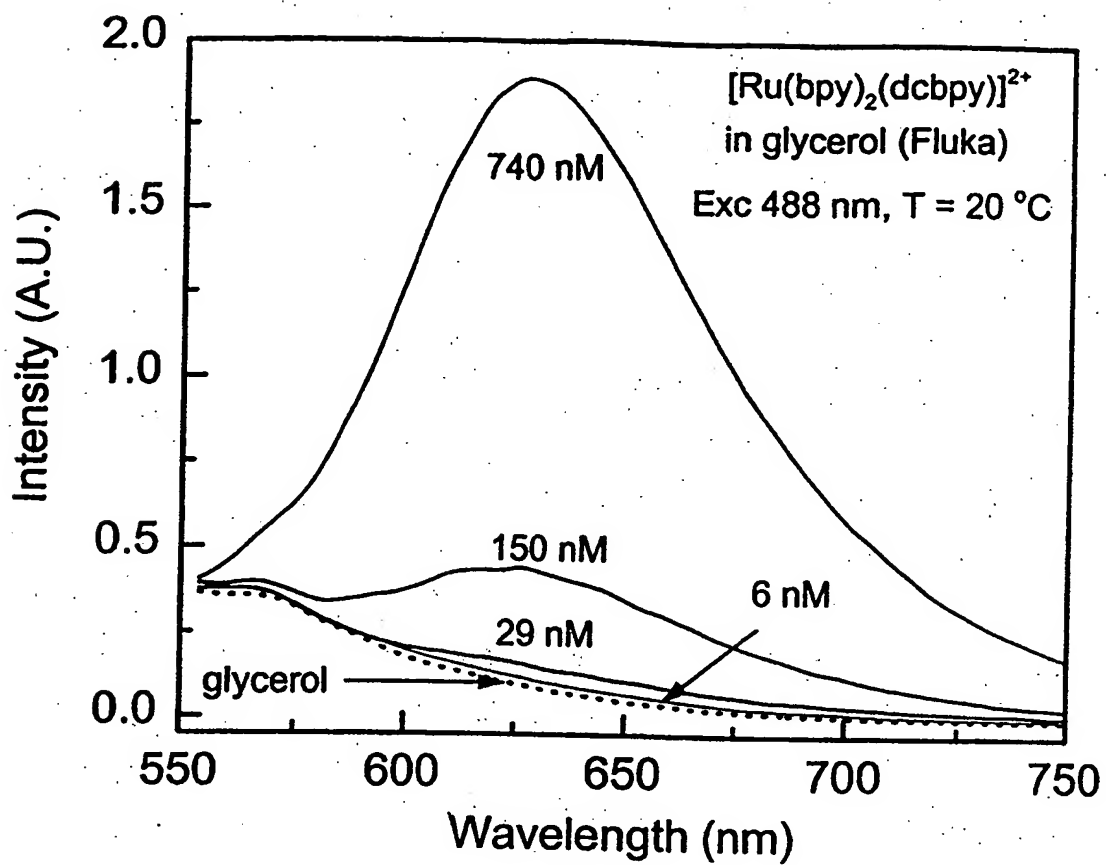


Fig. 8

14/20

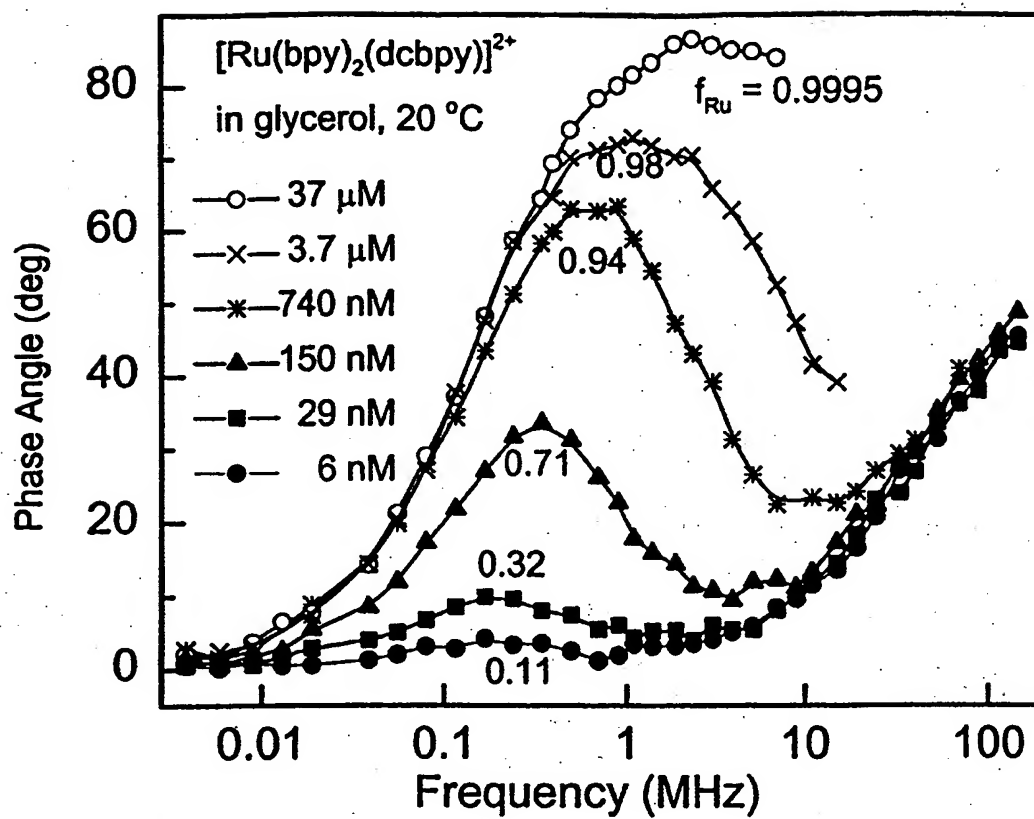


Fig. 9

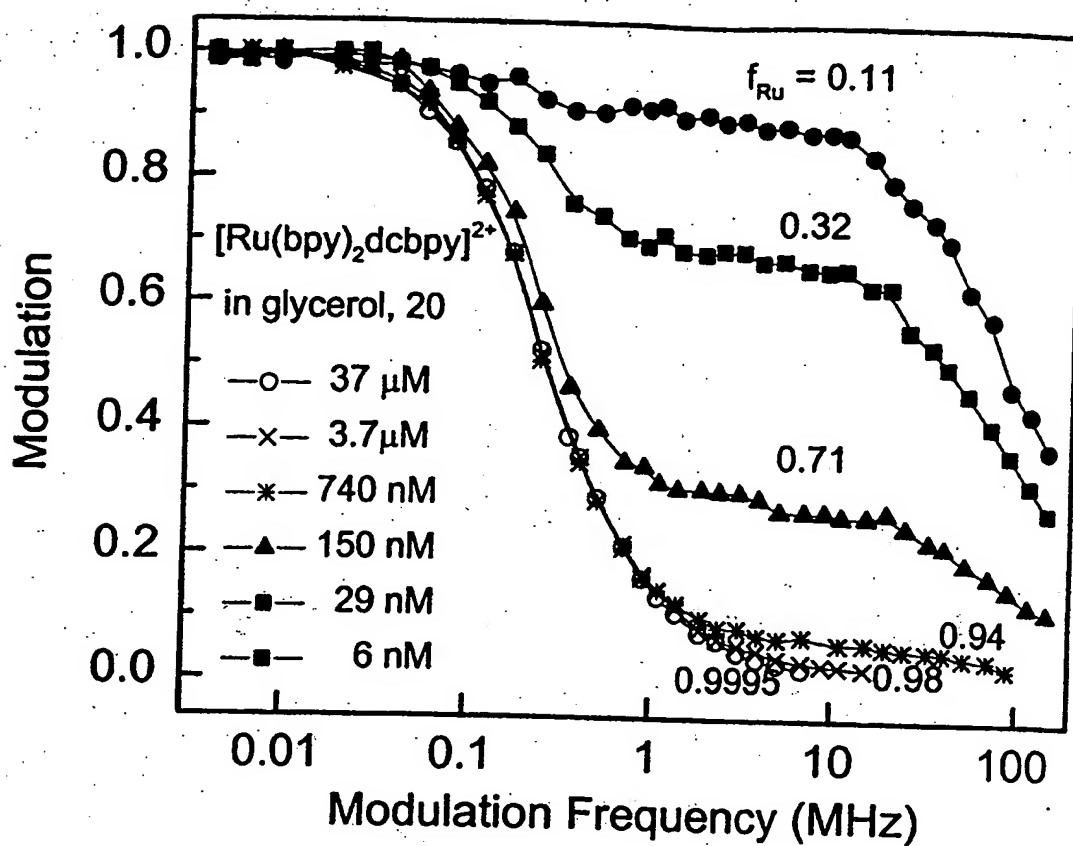


Fig. 10

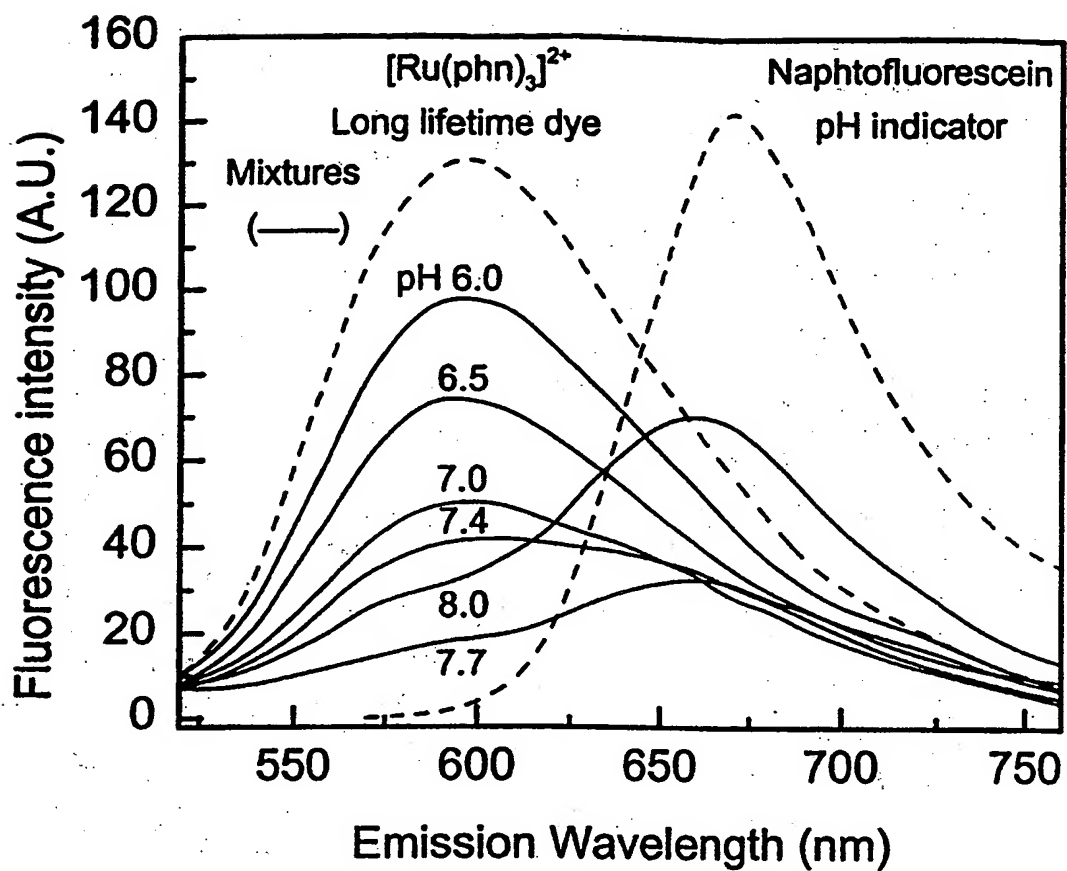


Fig. 11

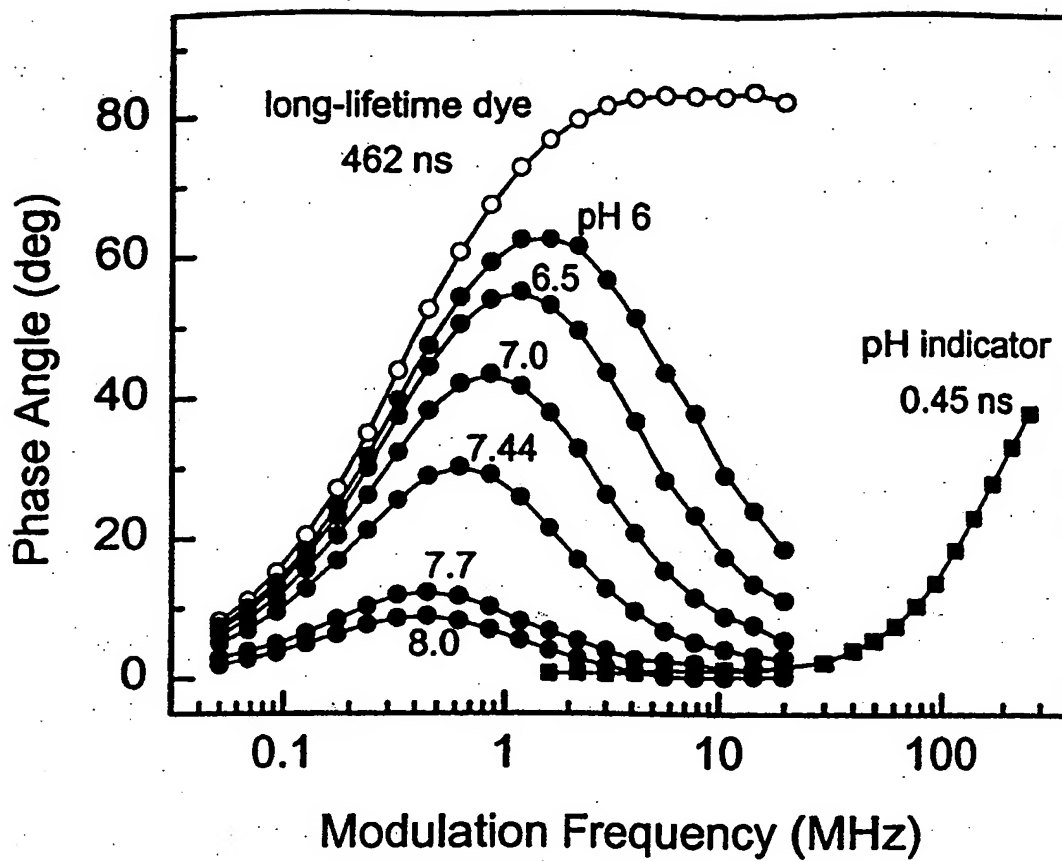


Fig. 12

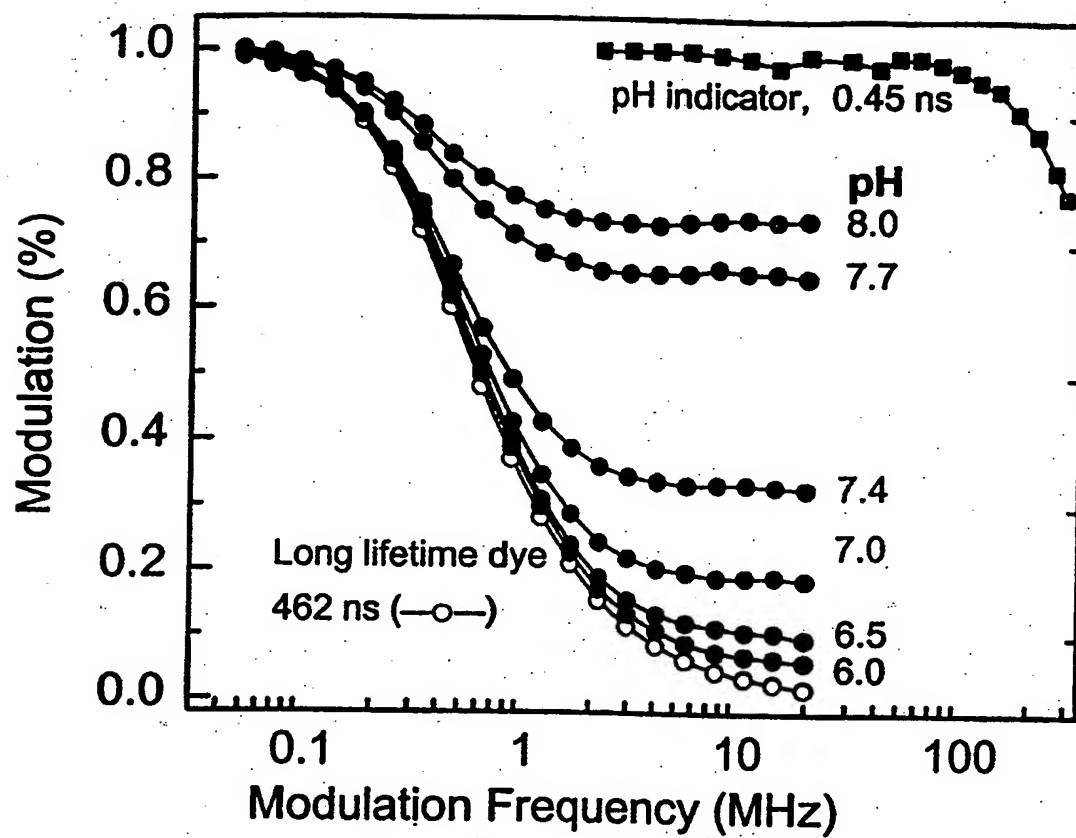


Fig. 13

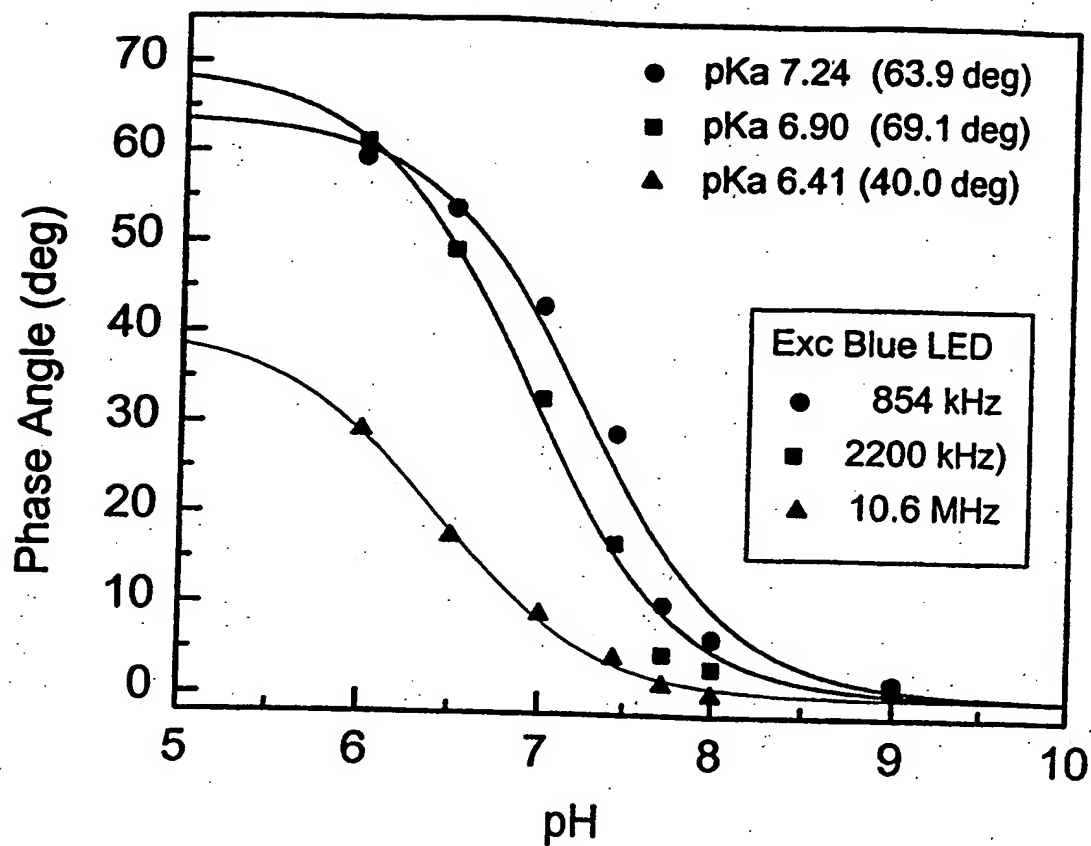


Fig. 14

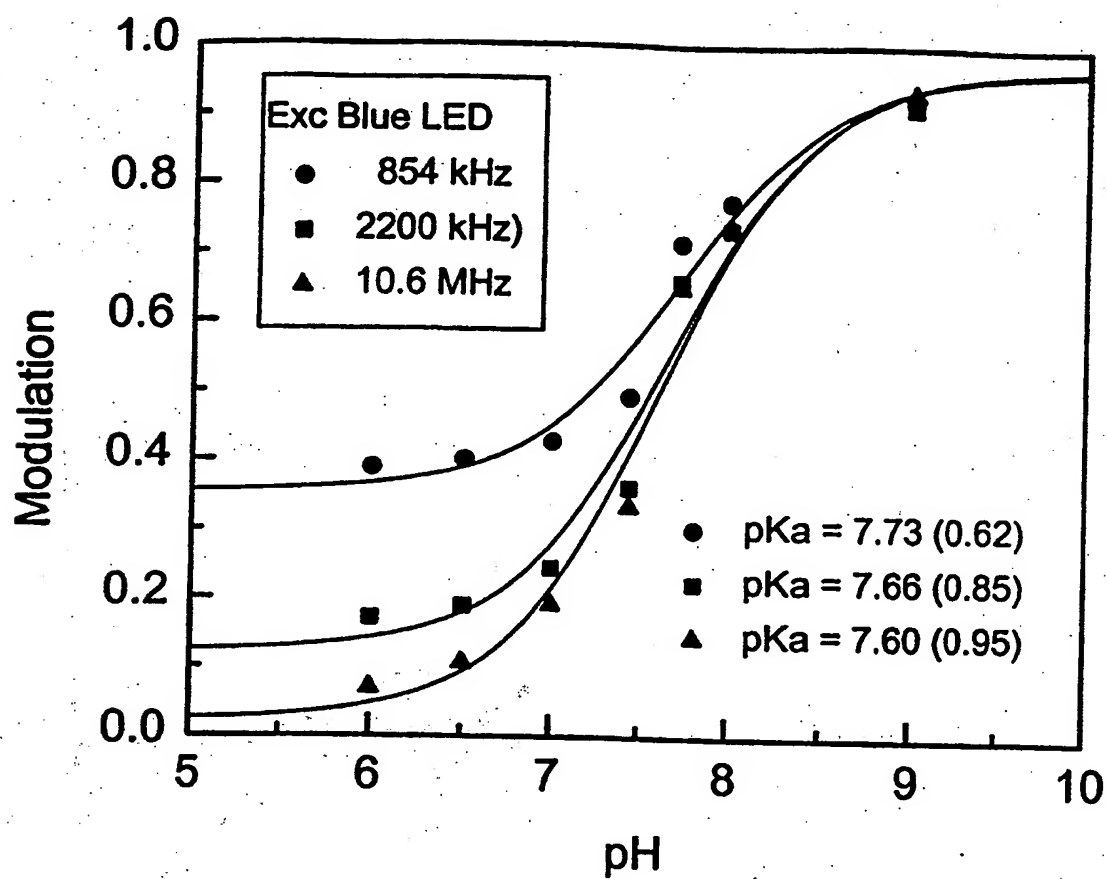


Fig. 15

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10874

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : G01N 21/64, 15/14

US CL : Please See Extra Sheet.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 250/461.2, 458.1, 461.1, 459.1, 461.2, 302; 436/172, 527, 521, 169, 501

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAPLUS, WPIDS, USPAT, DIALOG, MEDLINE
FLUORESC, PHASE, MODULATE, FREQUENT, PROBE, AMPLITUDE, LIFETIME, FLUORMET, POLYMERIC
MATRI.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 92/07245 A1 (THE UNIVERSITY OF MARYLAND) 30 April 1992, pages 3-43.	1-8
Y	WO 92/13265 A1 (THE UNIVERSITY OF MARYLAND) 06 August 1992, whole document, especially pages 9-48.	1-8
Y	US 5,270,548 A (STEINKAMP) 14 December 1993, whole document especially columns 3-10.	1-7
X	US 5,315,122 A (PINSKY et al) 24 May 1994, column 2, lines 59-68.	1-7

☐ Further documents are listed in the continuation of Box C.
 ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
A document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
B earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
O document referring to an oral disclosure, use, exhibition or other means	
P document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

04 AUGUST 1999

Date of mailing of the international search report

30 AUG 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231

Facsimile No. (703) 305-3230

Authorized officer

PADMA BASKAR

Telephone No. (703) 308-0196

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10874

A. CLASSIFICATION OF SUBJECT MATTER:

US CL :

250/461.2, 458.1, 461.1, 459.1, 461.2, 302; 436/172, 527, 521, 169, 501

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.

THIS PAGE BLANK (USPTO)